

# New insights into cell cycle control from the *Drosophila* endocycle

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During metazoan development, the organization of the cell cycle is often modified in response to developmental signals. The endocycle provides a dramatic example of this phenomenon. In the endocycle, also referred to as the endoreplicative cycle, cells undergo successive rounds of DNA replication without an intervening mitosis. Often the endocycle is used to expand the genome of a group of specialized cells that are highly biosynthetically active. In these circumstances, large polyploid cells are produced in organisms that are primarily comprised of diploid cells. However, many organisms achieve growth by increasing cell size, rather than cell number. This strategy is more generally exploited in insects and plants. For instance, in the insect *Drosophila melanogaster*, the majority of the larval tissues, as well as many adult tissues, enter the endocycle and become polyploid. Therefore, *Drosophila* has been a rich source for studies on endocycle regulation. Recent work from *Drosophila* is beginning to reveal how developmental signals promote the transition from the mitotic cycle to the endocycle, as well as what drives endocycle progression. In addition, studies on the endocycle have provided insight into the regulatory principles underlying the once per cell cycle replication of the genome, as well as the relationship between S phase and mitosis.

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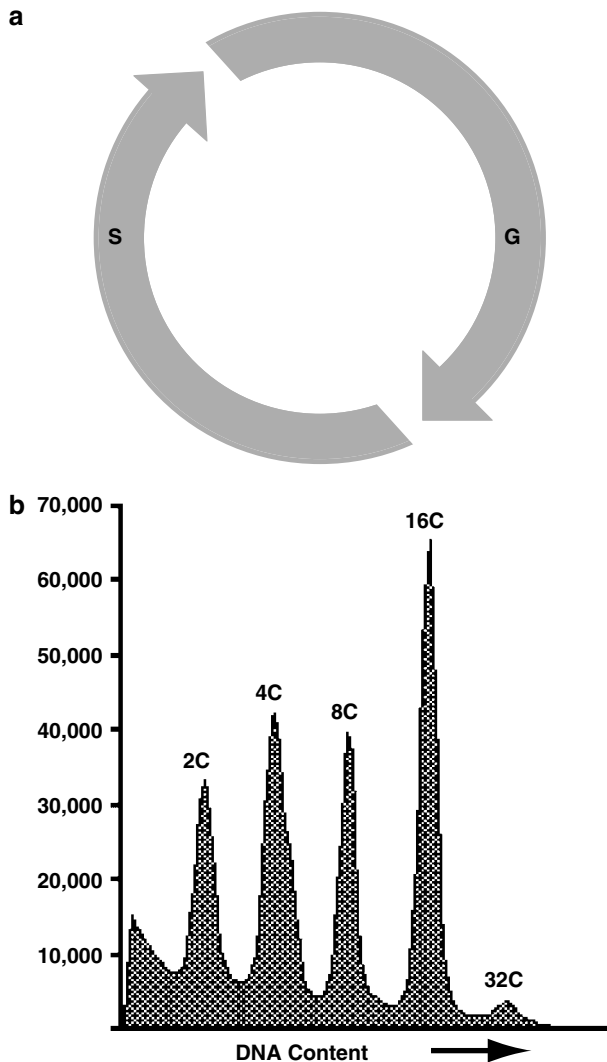
The endocycle is observed widely throughout both the plant and animal kingdoms. Indeed, the last several years have seen the publication of several excellent general reviews on the regulation of this common variant cell cycle (Zybina and Zybina, 1996; Traas *et al.*, 1998; Edgar and Orr-Weaver, 2001). Therefore, we have chosen to focus this review on recent findings on endoreplication in *Drosophila melanogaster*, with particular emphasis on the similarities and differences in the regulation of DNA replication in mitotic versus endocycling cells, as well as the molecular mechanism underlying the developmentally programmed mitotic/endocycle switch.

## The nature of the endocycle

In the endocycle, DNA replication is uncoupled from mitosis allowing cells to increase dramatically their DNA content above diploid values. At first glance, it may appear that endocycling cells do not adhere to the basic principles that apply to the regulation of the mitotic cycle. However, early studies on the endocycle hinted that many of the rules that governed DNA replication and the G<sub>1</sub>–S program during the mitotic cycle are enforced during the endocycle. One of the first observations suggesting this conservation of mechanism came from pulse-labeling studies using [<sup>3</sup>H]thymidine, which found that endocycling cells do not continuously replicate their DNA but, like mitotic cells, consist of alternating synthesis (S) phases and Gap (G) phases (King and Burnett, 1959; Balls and Billett, 1973; Hammond and Laird, 1985a, b). A subsequent comprehensive examination of endoreplication during embryogenesis determined that the cyclic alteration of S and G is a defining feature of all *Drosophila* endocycles (Smith and Orr-Weaver, 1991). Consistent with DNA replication being confined to a specific period or phase of the endocycle, cytophotometric studies and, more recently, flow cytometry (FACS) indicate that endocycling cells fall into discrete ploidy classes that approximate, but as explained below, are often slightly below true doublings of genomic DNA content (Figure 1) (Hammond and Laird, 1985a, b; Smith and Orr-Weaver, 1991; Lilly and Spradling, 1996). The simplest explanation for these data is that the re-replication controls that ensure that each DNA sequence is replicated only once per S phase in the mitotic cycle are operating in the endocycle. In support of this model, recent molecular genetic studies indicate that constraints imposed by the regulatory networks that license DNA replication origins require that cells in the endocycle undergo an obligate Gap phase between successive rounds of DNA replication (Follette *et al.*, 1998; Weiss *et al.*, 1998). Thus, the endocycle resembles a mitotic cycle in which S-phase controls are maintained, but cells are no longer obliged to undergo cellular division.

As our molecular understanding of endocycle regulation increases, it has become clear that the endocycle can be thought of as a modified mitotic cycle. The ability of the endocycle to skip mitosis likely reflects the modular nature and flexibility of the archetypal mitotic cell cycle

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**Figure 1** Cells in the endocycle undergo alternating S phases, Gap phases. (a) The *Drosophila* endocycle. (b) FACS profile of follicle cells from the *Drosophila* ovary. The number over each peak represents the genomic copy number of euchromatic sequences. Note that the follicle cells fall into distinct peaks, representing individual ploidy classes, confirming that DNA replication is not continuous but consists of discrete S phases and Gap phases

( $G_1$ –S– $G_2$ –M) and not the workings of an alternative mechanism to replicate the genome. The ordered progression of the cell cycle, with mitosis always following S phase, is driven by the cyclic accumulation and degradation of cyclins, the activating subunits of cyclin-dependent kinases (Cdks) (Nurse, 1994). In the mitotic cycle, the association of S-phase cyclins with Cdk2 promotes entry into S phase and DNA replication, while the activation of Cdk1 by the mitotic cyclins promotes entry into mitosis. In *Drosophila*, Cyclin E acts as the primary S-phase cyclin, while Cyclin A, Cyclin B, and Cyclin B3 function as mitotic cyclins (Lee and Orr-Weaver, 2003). Not surprisingly, as described in detail in the next section, Cyclin E/Cdk2 kinase activity is the primary Cyclin/Cdk combination driving endocycle progression in *Drosophila*.

Studies over the last 20 years have shown that while mitotic functions are repressed in the endocycle, many of the proteins required for DNA replication and the regulation of the  $G_1$ –S program are shared between the mitotic cycle and the endocycle (Edgar and Orr-Weaver, 2001; Lee and Orr-Weaver, 2003). Of particular importance is the conserved relationship between the licensing of DNA replication origins and Cdk activity. During the archetypal mitotic cycle, the once per cell cycle replication of DNA is achieved because two sequential steps in the process of DNA replication have opposite requirements for Cdk activity. While the formation of prereplication complexes (pre-RC), or licensing, requires the presence of low Cdk activity, the actual initiation of DNA replication is triggered by high Cdk activity (Bell and Dutta, 2002). Studies in numerous organisms provide a model in which pre-RCs form when Cdt1, known as Double-Parked (Dup) in *Drosophila*, acts with Cdc6 to load the MCM complex (MCM(2–7)) onto the origin. The MCM complex is the putative replicative helicase and is thought to facilitate the unwinding of the DNA. Once pre-RCs are assembled, the cell is licensed to initiate DNA replication when  $G_1$  cyclin activity achieves a threshold that triggers the  $G_1$ –S transition. After replication origins fire, they cannot reassemble until Cyclin/Cdk activity is once again low. In the mitotic cycle, this window of opportunity occurs in late mitosis after the destruction of the mitotic cyclins by the anaphase-promoting complex/cyclosome (APC/C) and in early  $G_1$ , before the accumulation of the  $G_1$  Cyclins. The mutually exclusive states of Cdk activity promoting licensing versus the firing of DNA replication origins ensure that each genomic sequence is replicated only once per cell cycle.

### Cyclin E/Cdk2: running a cell cycle engine with a single piston

Cyclin E and its associated kinase Cdk2 are required for DNA replication in *Drosophila* during both mitotic and endoreplicative cell cycles (Knoblich *et al.*, 1994; Lane *et al.*, 2000). In contrast, the mitotic cyclins are neither required nor expressed in most endocycling cells (Lehner and O'Farrell, 1990; Whitfield *et al.*, 1990; Stern *et al.*, 1993; Lilly and Spradling, 1996; Jacobs *et al.*, 1998). Cyclin D is expressed during the endocycle, but functions primarily in promoting growth and does not seem to have a direct role in promoting cell cycle progression (Datar *et al.*, 2000). Thus, the regulated accumulation and destruction of Cyclin E appears to be the primary force driving endocycle progression in *Drosophila*. Accordingly, the levels of Cyclin E oscillate during the endocycle (Lilly and Spradling, 1996; Royzman *et al.*, 1997; Weng *et al.*, 2003). The oscillations of Cyclin E suggest that a period of low Cyclin E/Cdk2 activity is required to relicense origins to allow successive endocycles. Consistent with this idea, the continuous overexpression of Cyclin E from a transgene

in larval salivary glands blocks endocycle progression, resulting in small salivary glands with nuclei that have undergone little DNA replication (Follette *et al.*, 1998; Weiss *et al.*, 1998). Similarly, the continuous over-expression of Cyclin E in the ovarian follicle cells blocks polyploidization (Calvi *et al.*, 1998; Shcherbata *et al.*, 2004). These results strongly suggest that the inhibitory effects of Cyclin/Cdks on pre-RC formation observed during the mitotic cycle are conserved in the endocycle. Thus, the obligate Gap phase of the endocycle is the result of the conserved requirement for low Cdk activity to reset DNA replication origins.

The *Drosophila* endocycle appears to be the simplest of cell cycles, driven by the oscillations of a single Cyclin/Cdk combination, Cyclin E/Cdk2. Yet, how are the oscillations of Cyclin E/Cdk2 activity achieved during the endocycle? While a complete answer to this question is not currently available, the majority of the evidence points to the importance of the cyclic accumulation and destruction of the Cyclin E protein. During the endocycle, the periodicity of Cyclin E expression is influenced by multiple inputs. Intriguingly, the importance of any one input in defining the Cyclin E oscillator may be cell type specific. The Skp1-Cull1-F-box (SCF) protein complex, which functions as an E3 ubiquitin ligase, targets the Cyclin E protein for proteolysis (Koepp *et al.*, 2001; Moberg *et al.*, 2001; Strohmaier *et al.*, 2001). The F-box component of the SCF provides substrate specificity. Archipelago (Ago), known as hCdc4 or Fbw7 in mammals, is the F-box protein that physically interacts with Cyclin E (Koepp *et al.*, 2001; Moberg *et al.*, 2001; Strohmaier *et al.*, 2001). In *ago* mutants, Cyclin E protein accumulates in both mitotic and endocycling cells. Persistent Cyclin E accumulation has negative effects on endocycle progression, resulting in phenotypes ranging from a lengthening of the S phase in the polyploid nurse cells to a complete abrogation of endoreplication in the somatic follicle cells of the ovary (Doronkin *et al.*, 2003; Shcherbata *et al.*, 2004). The COP9 signalosome positively regulates the activity of the SCF complex and promotes the degradation of Cyclin E (Doronkin *et al.*, 2003). Mutations in two components of the COP9 signalosome, *CSN4* and *CSN5*, alter the kinetics of Cyclin E oscillations as well as the dynamics of the S–G cycle in polyploid nurse cells in a manner similar to that observed in *ago* mutants. In mammals, the Cyclin E/Cdk2-dependent phosphorylation of Cyclin E promotes ubiquitin-dependent destruction of the Cyclin E protein (Clurman *et al.*, 1996; Won and Reed, 1996). Thus, Cyclin E is predicted to regulate negatively its own abundance. Data from the hypomorphic mutant *cyclin E*<sup>01672</sup> suggests that a similar autoregulatory mechanism may be operating in *Drosophila* (Lilly and Spradling, 1996). The oscillations of Cyclin E protein are severely dampened in *cyclin E*<sup>01672</sup> polyploid nurse cells such that endocycling nuclei are rarely observed to have extremely high or extremely low levels of Cyclin E protein. While the exact cause of the reduced oscillations is not clear, one model is that in *cyclin E*<sup>01672</sup> nurse cells, the reduced accumulation of Cyclin E results in diminished ability to carry out the

rapid destruction of the Cyclin E protein at the end of each endocycle S phase (Lilly and Spradling, 1996). Thus, as has been demonstrated in mitotic cycles, an important component of the periodic accumulation of Cyclin E during the endocycle is likely to be an autoregulatory loop, which couples Cyclin E/Cdk2 activity to the regulated proteolysis of Cyclin E protein via the SCF.

E2F transcription factors, consisting of a heterodimer of E2F1 and DP proteins, can act as either transcriptional activators or transcriptional repressors depending on the nature of the E2F subunit (DeGregori, 2002). In *Drosophila*, E2F1 and DP form a heterodimeric transcription factor that activates a transcriptional program of S-phase genes that includes Cyclin E as well as *RNR2* and *PCNA*, two genes required for DNA replication (Duronio *et al.*, 1995, 1998; Royzman *et al.*, 1997). Intriguingly, Cyclin E negatively regulates this transcriptional program during embryonic endocycles (Duronio and O'Farrell, 1995; Sauer *et al.*, 1995). Thus, the necessary elements are present for a negative feedback loop in which Cyclin E negatively regulates its own transcription via the downregulation of E2F1 activity. During embryogenesis, this negative feedback loop ensures that the accumulation of Cyclin E transcripts in endocycling cells peaks prior to S-phase entry (Duronio and O'Farrell, 1995; Sauer *et al.*, 1995). However, the analysis of *E2f1* and *Dp* mutants indicate that this regulatory loop does not play an essential role in the regulation of the G–S cycle during embryonic endocycles. Specifically, in *E2f1* and *Dp* mutants, endoreplication proceeds in the absence of the cyclic accumulation of transcript (Royzman *et al.*, 1997). Presumably, the basal levels of Cyclin E expression as well as maternally loaded Cyclin E are sufficient to support embryogenesis and most of larval development. These data are consistent with the observation that Cyclin E protein oscillations occur in the absence of the cyclic accumulation of the Cyclin E transcript in the polyploid nurse cells of the ovary (Royzman *et al.*, 2002). While the E2F1-dependent accumulation of Cyclin E transcript is not an absolute requirement for endocycle progression, it may help sharpen Cyclin E protein oscillations during embryonic and larval endocycles. That this is functionally important is supported by the observation that the pattern of embryonic endocycles is altered in *E2f1* and *Dp* mutants in a manner suggesting that the endo-S phase has a longer duration (Duronio *et al.*, 1998). Mutation of the genes encoding Cyclin D/Cdk4, a positive regulator of E2F, causes a similar phenotype (Meyer *et al.*, 2002b; Emmerich *et al.*, 2004). Moreover, the loss of E2F repressor function in larval endocycles results in continuous rather than cyclic expression of cyclin, which ultimately inhibits the endocycle (Weng *et al.*, 2003). Thus, E2F-directed transcription of *Cyclin E* contributes to the integrity of the endocycle in some, but not all cell types.

As indicated above, both transcriptional and post-transcriptional inputs drive the oscillation of Cyclin E protein, and thus Cyclin E/Cdk2 activity during the endocycle in *Drosophila*. However, it has recently been proposed that the periodic inhibition of Cyclin E/Cdk2

activity by the p27<sup>CIP/KIP</sup> like cyclin-dependent kinase inhibitor (CKI) Dacapo (Dap) influences the kinetics of the S–G cycle during endoreplication (de Nooij *et al.*, 2000; Edgar and Orr-Weaver, 2001; Hong *et al.*, 2003). Specifically, Dap is proposed to inhibit transiently Cyclin E/Cdk2 activity to promote entry into the Gap phase. Dap binds and specifically inhibits Cyclin E/Cdk2 complexes in *Drosophila* (de Nooij *et al.*, 1996; Lane *et al.*, 1996). In the polyploid nurse cells of the ovary, Dap oscillations closely follow those of Cyclin E (de Nooij *et al.*, 2000). In addition, in many tissues of *Drosophila* Cyclin E positively influences the accumulation of Dap (de Nooij *et al.*, 2000). This suggests a feedback loop in which a rise in Cyclin E levels triggers DNA replication, as well as the accumulation of Dap. Eventually, Dap levels may rise high enough to inhibit Cyclin E/Cdk2 activity, thus halting Sphase and introducing a Gap phase. In support of this model, endoreplication is compromised in *dap* mutant nurse cells resulting in nuclei with inappropriately low DNA contents. A similar mechanism may be operating during endoreplication in mammalian trophoblasts where Cyclin E levels remain continuously elevated. In trophoblasts, endocycle progression is accompanied by the oscillations of the CIP/KIP family member p57 (Hattori *et al.*, 2000). Mirroring the temporal distribution of Dap in endocycling nurse cells, in endocycling trophoblasts p57 accumulates at the end of each Sphase and is destroyed prior to the subsequent Sphase. Thus, the oscillation of CIP/KIP-like CKIs may be a common feature of endocycles in diverse organisms.

While Cyclin E plays a central role in the regulation of DNA replication in both mitotic and endocycling cells, there are some intriguing differences in how these different cycles respond to Cyclin E. For example, the persistent overexpression of Cyclin E has very different effects on mitotic versus endocycling cells. While the overexpression of Cyclin E blocks endocycle progression in the polytene nuclei of the larval salivary gland, it promotes mitotic progression in the proliferating cells of the wing imaginal disc (Follette *et al.*, 1998; Neufeld *et al.*, 1998; Weiss *et al.*, 1998). Similarly, in the diploid cells of the eye imaginal disc, *ago* mutant clones have a growth advantage relative to a wild-type twin spot, indicating that cells lacking Ago divide more quickly than wild type (Moberg *et al.*, 2001). In contrast, in the follicle cells of the ovary, *ago* mutant clones complete the mitotic cycles but fail to undergo endoreplication (Shcherbata *et al.*, 2004). The compromised endoreplication observed in *ago* mutants is likely due to the accumulation of the Cyclin E protein, although it cannot be ruled out that *ago* affects critical targets in addition to Cyclin E. It is not clear why endocycling cells might be more sensitive to the persistent expression of Cyclin E relative to cells in the mitotic cycle. However, it has been suggested that the nuclear envelope break down in the mitotic cycle, which does not occur during the endocycle in *Drosophila*, might transiently lower Cyclin E/Cdk2 activity allowing pre-RC assembly (Edgar and Orr-Weaver, 2001). A second model is suggested by the observation that *ago* mutant

follicle cells fail to fully downregulate the mitotic cyclins prior to endocycle entry. Thus, inappropriately high Cyclin E levels may compromise the mitotic/endocycle switch (Shcherbata *et al.*, 2004).

Another notable difference between the roles of Cyclin E in mitotic versus endocycling cells involves the licensing of DNA replication origins. The rapid and transient induction of Cyclin E expression using a heat-inducible cyclin E transgene results in the almost immediate loading of the MCM complex onto the chromatin in the polytene nuclei of the larval salivary gland, an effect that is not observed when Cyclin E is overexpressed in mitotically active cells (Su and O'Farrell, 1997, 1998). Intriguingly, Cyclin E appears to play a similar role in promoting MCM loading in mammalian cells that re-enter the cell cycle from G<sub>0</sub> (Geng *et al.*, 2003; Parisi *et al.*, 2003). Cyclin E-deficient mouse embryonic fibroblasts, rendered quiescent through serum starvation, cannot re-enter the cell cycle upon the readdition of serum (Geng *et al.*, 2003; Parisi *et al.*, 2003). The inability to transit from G<sub>0</sub> to S correlates with the failure to load the MCMs. It has been proposed that under certain circumstances, including re-entry into the cell cycle from quiescence, Cyclin E is required to open a 'window of opportunity' for MCM loading (Coverley *et al.*, 2002).

A special role for Cyclin E during endoreplication is suggested by the phenotype of Cyclin E1<sup>-/-</sup>, E2<sup>-/-</sup> double knockout mice (Geng *et al.*, 2003; Parisi *et al.*, 2003). Unlike *Drosophila* where Cyclin E is required for DNA replication during the mitotic cycle, Cyclin E-deficient mice display surprisingly few disruptions in cell cycle regulation. However, the endocycle provides a significant exception (Geng *et al.*, 2003; Parisi *et al.*, 2003). In Cyclin E-deficient mice, endoreplication in both the giant trophoblasts of the placenta and in megakaryocytes is severely disrupted. The specific function of Cyclin E during the mammalian endocycle is unknown. However, one attractive model is that, as observed in *Drosophila*, Cyclin E is required to load the MCM complex onto the origin during endocycles. What features might cells undergoing endoreplication and cells re-entering the cell cycle from G<sub>0</sub> share that might explain this unique function of Cyclin E? It is interesting that cells transitioning from G<sub>0</sub> to S as well as cells in the endocycle do not enter the Sphase from mitosis, as occurs during the archetypal mitotic cycle. Thus, this unique role for Cyclin E may derive from the presence or absence of mitotic cyclins and/or the status of the APC/C. Future studies on the role of Cyclin E in mitotic versus endocycling cells are likely to provide general insights into the licensing of replication origins and the regulation of DNA replication.

#### Under the radar: How do endocycling cells bypass checkpoint controls?

While there are many similarities in the regulation of the G–S cycle in mitotic and endocycling cells, there are also

very clear differences. One of the most intriguing differences is how mitotic versus endocycling cells respond to incomplete DNA replication. In the endocycle, Sphase is often truncated before the entire genome is replicated. This truncation, or early entry into the Gap phase prior to the completion of DNA reduplication, results in the under-representation of late replicating heterochromatic sequences in many polyploid cell types (Gall *et al.*, 1971; Hammond and Laird, 1985a, b; Lilly and Spradling, 1996). Previous work has shown that the degree of S-phase truncation is influenced by the kinetics of Cyclin E oscillations (Lilly and Spradling, 1996; Doronkin *et al.*, 2003). When the kinetics of Cyclin E oscillations are altered, for example, by overexpressing Cyclin E from a transgene, Sphase proceeds to apparent completion and the entire genome is replicated including late replicating heterochromatin (Lilly and Spradling, 1996; Leach *et al.*, 2000). Similarly, mutations in genes required for the degradation of Cyclin E protein lead to an increase in the copy number of heterochromatic sequences in polyploid nurse cells (Doronkin *et al.*, 2003). These data suggest that Cyclin E is continuously required during the S phase to support DNA replication in endocycles and that the Cyclin E oscillator is not coupled to S-phase completion. Thus, once Cyclin E levels fall below that which will support DNA replication, S phase stops and cells enter the Gap phase with unreplicated DNA.

In the mitotic cycle, blocks entry into mitosis if a cell has not completed genomic replication (Hartwell and Weinert, 1989; Nurse, 1994). The S–M checkpoint acts through the mitotic cyclins and Cdk1 and, therefore, is not predicted to be operative in most polyploid cell types, which do not express mitotic cyclins. However, it is curious that the presence of unreplicated DNA in endocycling cells of *Drosophila* does not appear to trigger an intra-S-phase checkpoint. In both mammals and yeast, the intra-S-phase checkpoint monitors DNA for damage, including the presence of stalled or collapsed replication forks, during the S phase (Nurse, 1994; Paulovich and Hartwell, 1995; Diffley *et al.*, 2000; Tercero *et al.*, 2003; Bartek *et al.*, 2004). In mammals, the activation of the intra-S-phase checkpoint slows S-phase progression and inhibits the firing of late replication origins (Nyberg *et al.*, 2002; Bartek *et al.*, 2004). During the endocycle, polyploid cells repeatedly enter the S phase and replicate their DNA in the presence of large numbers of stalled and/or collapsed replication forks (Leach *et al.*, 2000). These forks are predicted to be present because, as described above, endocycling cells often truncate S phase leaving up to 20% of their genome unreplicated. How do endocycling cells ignore this tremendous genomic insult? One possibility is that *Drosophila* simply does not have an intra-S-phase checkpoint. This seems unlikely given that an intra S-phase checkpoint is found in both yeast and mammals (Nyberg *et al.*, 2002; Sancar *et al.*, 2004). In addition, irradiating proliferating *Drosophila* imaginal disc cells decreases the rate of BrdU incorporation consistent with an intra-S-phase checkpoint operating during the mitotic cycle (Jaklevic

and Su, 2004). Perhaps as occurs with the mitotic machinery, key components of the intra-S-phase checkpoint pathway are not expressed in endocycles. Alternatively, polyploid cells may bypass the intra-S-phase checkpoint via a novel mechanism that is specific to the endocycle.

How might cells in the endocycle bypass the intra-S-phase checkpoint? In mammals, the activation of the intra-S-phase checkpoint via the upstream signaling kinases, ATM or ATR, slows progression through Sphase by negatively regulating the stability of the phosphatase Cdc25A, an activator of Cdk2 (Bartek *et al.*, 2004). This slowing of S phase works in concert with the inhibition of late origin firing to allow cells time to repair DNA damage before entering mitosis. Yet, in *Drosophila*, Cdc25 is apparently not required for the activity of Cdk2 (Lane *et al.*, 2000). Thus, the slowing of S phase observed in mitotic cells after irradiation must work through an alternative mechanism. Perhaps, the intra-S-phase checkpoint of *Drosophila* acts primarily by inhibiting the firing of late origins of DNA replication. As described above, the S-phase truncation model predicts that in most endocycling cells Cyclin E levels fall below the threshold to support DNA replication before late origins are programmed to fire. This truncation might make endocycling cells impervious to the inhibition of late origin firing imposed by the intra-S-phase checkpoint. Thus, an intra-S-phase checkpoint may be activated by the presence of numerous stalled replication forks in most endocycling cells, but this may not effect cell cycle progression. In contrast, in a mitotic cell, the inhibition of late origin firing would stall cell cycle progression because the S–M checkpoint ensures that a cell will not enter mitosis with unreplicated DNA.

There is limited evidence that endocycling cells do recognize the presence of stalled replication forks and/or DNA damage caused by the truncation of Sphase. In *Drosophila*, the variant histone H2Av is phosphorylated on its C-terminal tail in response to the presence of a double-stranded break (Madigan *et al.*, 2002). Phosphorylated H2Av is referred to as  $\gamma$ -H2Av. In the somatic follicle cells, a dramatic increase in  $\gamma$ -H2Av is observed upon entry into the endocycle and the completion of the first truncated Sphase. These data suggest that stalled forks and/or DNA damage are recognized during the endocycle, but this signal is either not properly transduced or is otherwise not sufficient, to slow S-phase progression. Understanding how endocycling cells replicate their DNA in the presence of stalled replication forks and/or other DNA damage associated with under-replication may increase our understanding of the molecular mechanisms that control the intra-S-phase checkpoint during the mitotic cell cycle, as well as how cancer cells evade checkpoint controls.

#### Under-replication as a model for studying chromatin structure and origin timing

The characterization of mutants that alter the replication of heterochromatin during the endocycle have

provided unique insights into the regulation of chromatin structure. In hypomorphic female sterile *E2f1* and *Dp* mutants, the polyploid nurse cells of the ovary inappropriately replicate their heterochromatin independent of a notable disruption of the kinetics of Cyclin E protein oscillations or a decrease in the levels of *cyclin E* transcription (Royzman *et al.*, 2002). One potential explanation for this observation is that E2F1 and DP have functions that are independent of their transcriptional activities (Royzman *et al.*, 1999; Bosco *et al.*, 2001). These functions may involve maintaining the chromatin structure required to inhibit the firing of replication origins in heterochromatin until late in S phase (Royzman *et al.*, 2002). E2F1, DP and Rb are associated with chromatin at the chorion loci where they have been proposed to limit DNA amplification through an interaction with ORC (Bosco *et al.*, 2001). Thus, E2F1 and DP have previously been suggested to restrict origin usage. Indeed, recent work on the regulation of chorion gene amplification implicates the regulation of chromatin structure by complexes and proteins previously associated with transcriptional inhibition in the regulation of origin usage (Beall *et al.*, 2002; Aggarwal and Calvi, 2004; Korenjak *et al.*, 2004; Lewis *et al.*, 2004). Similar to what is observed with *E2f1* and *Dp* mutants in the ovary, the product of the suppressor of under-replication (SuUR) gene influences the degree to which heterochromatin is under-replicated in polytene salivary glands (Belyaeva *et al.*, 1998). SuUR encodes a protein that contains significant similarity to the ATP-binding domain of SNF2/SWI2 (Makunin *et al.*, 2002). The SuUR protein binds late replicating DNA as well as centric and intercalary heterochromatin in polytene chromosomes. In a SuUR mutant, intercalary and pericentric heterochromatin are more fully replicated. Moreover, the overexpression of SuUR leads to the spreading of under-replicated regions within polytene chromosomes. Future studies will determine if the regulation of late origin firing ascribed to E2F, DP and SuUR in endocycles, reflects a more general role for these proteins in defining chromatin structure and/or utilization of replication origins during the S phase in all cells.

### Entry into the endocycle: reigning in mitotic cyclin activity

How does a cell switch from a mitotic cycle to an endocycle? The observation that diploid cells can be shunted into an inappropriate endocycle by inhibiting mitotic cyclin activity suggests a possible mechanism (Sauer *et al.*, 1995; Hayashi, 1996; Weigmann *et al.*, 1997). In *Drosophila*, entry into mitosis is dependent on Cdk1 and the mitotic cyclin, Cyclin A (Lehner and O'Farrell, 1989; Stern *et al.*, 1993). Mutations in either *cyclin A* or *Cdk1* result in cells that, while developmentally programmed to be in the mitotic cycle, enter the endocycle and become polyploid. These artificially induced endocycles closely resemble developmentally

programmed endocycles. For example, impairing Cdk1 activity forces mitotically active optic lobe cells of the developing brain into the endocycle, which ultimately results in the production of nuclei containing large banded polytene chromosomes similar to the polytene nuclei of the larval salivary glands (Hayashi, 1996). Two conclusions can be drawn from these results. First, as has also been shown in other systems, in *Drosophila* the checkpoint that ensures the obligate oscillations of S phase and mitosis depends on Cdk1 and the mitotic cyclins. Second, a self-sustaining endocycle can be achieved, outside of the appropriate developmental context, by simply removing mitotic cyclin activity. Thus, the building blocks of an endocycle can be found within the regulatory networks of the mitotic cycle. However, we note that this observation does not preclude the existence of genes that have evolved specifically to promote the endocycle, although none have thus far been identified.

How are the mitotic cyclins downregulated during a developmentally programmed mitotic/endocycle transition, and is this mechanism conserved between cell types? Entry into the endocycle is accompanied by the transcriptional downregulation of many genes that promote mitotic progression including *cyclin A*, *cyclin B*, *cyclin B3*, *Cdk1* and *Cdc25/string* (Smith and Orr-Weaver, 1991; Sauer *et al.*, 1995; Schaeffer *et al.*, 2004). However, it is unlikely that this transcriptional inhibition is sufficient to trigger the mitotic/endocycle switch. Several lines of evidence indicate that the proteolysis of the mitotic cyclins is essential for entry into a developmentally programmed endocycle. The mitotic cyclins are degraded by the highly conserved APC/C (Sigrist and Lehner, 1997; Schaeffer *et al.*, 2004). The APC/C is an E3 ubiquitin ligase that targets proteins for destruction by the 26S proteasome. Fizzy-related (Fzr) is a Cdh1-like regulatory subunit of the APC/C that promotes the degradation of the mitotic cyclins in G<sub>1</sub> and is absolutely required for endocycles (Sigrist and Lehner, 1997; Jacobs *et al.*, 2002). Overexpression of *fzr* downregulates Cyclin A, Cyclin B and Cyclin B3, and inhibits mitosis. A role for Fzr in directing cells into the endocycle was first demonstrated in the well-studied polytene nuclei of the larval salivary gland and has since been extended to other tissues (see below) (Sigrist and Lehner, 1997; Schaeffer *et al.*, 2004). Salivary gland nuclei from third instar larvae contain large polytene chromosomes that consist of over 2000 copies of the haploid genome. The cells of the salivary gland first enter the endocycle during embryogenesis, after the completion of mitosis 16 at a time when no mitotic cyclins are detected (Smith and Orr-Weaver, 1991). *Fzr* is transcriptionally upregulated in the salivary gland at the time of the mitotic/endocycle switch (Sauer *et al.*, 1995). In *fzr* mutant embryos, mitotic cyclins are present at high levels in the salivary gland after the completion of mitosis 16, as well as in other tissues slated to enter the endocycle (Sigrist and Lehner, 1997). Not surprisingly, *fzr* mutants fail to enter developmentally programmed endocycles. However, even though the *fzr* mutant cells accumulate

mitotic cyclins, they do not precede into mitosis but arrest in G<sub>2</sub>. The observation that the accumulation of mitotic cyclins leads to a cell cycle arrest, and not re-entry into the mitotic cycle, suggests that the destruction of the mitotic cyclins through the upregulation of *fzr* is only one of several mechanisms that act in parallel to inhibit mitosis at the mitotic/endocycle boundary.

By degrading the mitotic cyclins, the APC/C inhibits Cdk1 activity and helps divorce the G<sub>1</sub>-S from the G<sub>2</sub>-M cycle, thus promoting endocycle entry. However, does the requirement for APC/C activity extend beyond the mitotic/endocycle boundary or is the transcriptional downregulation of mitotic activators sufficient to block re-entry into mitosis and ensure endocycle progression? Recent evidence suggests that the precise requirement for APC activity during the endocycle may be cell type specific (Kashevsky *et al.*, 2002). In the polyploid nurse cells of the *Drosophila* ovary, a transient increase in mitotic activity is thought to initiate a developmental alteration in chromatin structure and nuclear organization (Reed and Orr-Weaver, 1997; Kashevsky *et al.*, 2002). During the first four nurse cell endocycles, the chromosomes are partially polytene with all chromatids loosely aligned (Smith and Orr-Weaver, 1991; Dej and Spradling, 1999). However, after the completion of the fifth endocycle Sphase, the 32-chromatid pairs dissociate. Thus, the chromosomes go from being polytene, where sister chromatids remain aligned, to a more dispersed polyploid configuration. This transition from polyteny/polyploidy may facilitate the construction of the unusual dispersed nucleolus of the *Drosophila* nurse cells and has been proposed to involve the transient reactivation of the mitotic cyclins (Smith and Orr-Weaver, 1991; Dej and Spradling, 1999; Kashevsky *et al.*, 2002). Consistent with this idea, in an endocycle that is artificially induced by downregulating mitotic cyclins, chromatids remain tightly aligned (polytene) (Vidwans *et al.*, 2002).

*Morula (mr)* encodes the APC subunit, APC2 (Kashevsky *et al.*, 2002). In the hypomorphic female sterile mutants *mr<sup>1</sup>* and *mr<sup>2</sup>*, the polyploid nurse cells of the ovary undergo four seemingly normal endocycles before constructing large spindles and arresting in a metaphase-like state during the fifth endocycle (Reed and Orr-Weaver, 1997). These dramatic events are accompanied by the unscheduled accumulation of the mitotic cyclin, CycB. These data support a model in which the APC/C restrains nurse cells from fully entering the mitotic cycle after the brief burst of mitotic activity in the fifth endocycle. Importantly, stronger mutations in *mr* do not lead to an earlier onset of the phenotype, indicating that it is only during the unusual fifth endocycle, at the time of the polyploid/polytene transition, that the nurse cells are susceptible to being drawn back into the mitotic cycle. In addition, overexpression of the CycB protein during the first four nurse cell endocycles is not sufficient to block endocycle progression. These data suggest that during the first four endocycles, mitotic activity is suppressed via other mechanisms. This could include, for example, the

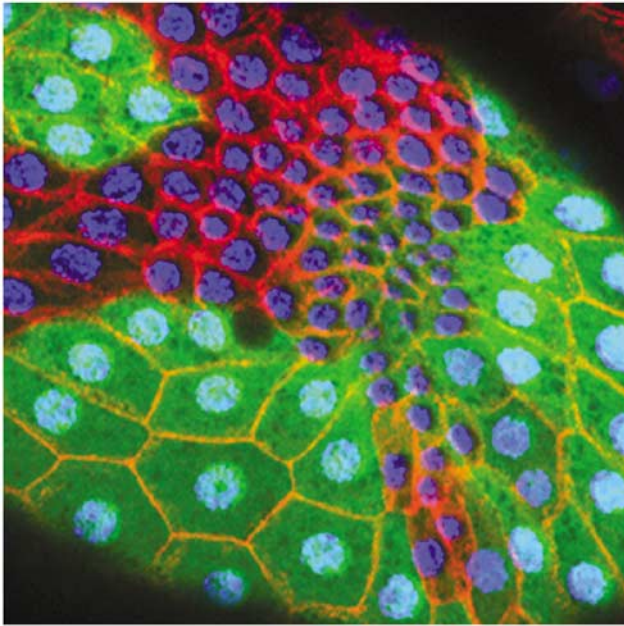
transcriptional downregulation of *Cdk1* or *string/Cdc25*. Thus, the exact requirement for the APC/C in promoting endocycle progression may depend on developmental context.

### Notch signaling and the mitotic/endocycle switch

In-depth studies on the mitotic/endocycle switch in the follicle cells of the ovary have tied the downstream events of endocycle entry to a specific upstream signaling pathway (Deng *et al.*, 2001; Lopez-Schier and St Johnston, 2001). As the endocycle can be followed simultaneously in two independent cell types, *Drosophila* oogenesis provides a useful model to examine questions concerning endocycle regulation. *Drosophila* oogenesis takes place in a 16-cell interconnected germline cyst (reviewed in de Cuevas *et al.*, 1997). Only one cell from the cyst commits to meiosis and forms the oocyte. The other 15 cells in the cyst enter the endocycle and develop as highly polyploid nurse cells that synthesize and deliver gene products to the growing oocyte. A layer of somatically derived follicle cells surrounds individual ovarian cysts. Together, the ovarian cysts and the overlying follicle cells comprise an egg chamber. The follicle cells divide mitotically and increase in number until mid-oogenesis (stage 6) when they uniformly exit the mitotic cycle and enter the endocycle. Recent work indicates that the Notch/Delta signaling pathway controls the mitotic/endocycle switch in follicle cells (Deng *et al.*, 2001; Lopez-Schier and St Johnston, 2001).

Notch is a large transmembrane receptor protein that interacts with the ligands Delta and Serrate (Artavanis-Tsakonas *et al.*, 1999). Removing Delta from the germ line, or Notch from the follicle cells, leads to the failure of the follicle cells to undergo the mitotic/endocycle transition (Deng *et al.*, 2001; Lopez-Schier and St Johnston, 2001). Additionally, the transcription factor Suppressor of Hairless (Su(H)), a downstream component of the Notch signaling pathway, is required cell autonomously in the follicle cells for endocycle entry (Figure 2). This demonstrates that some of the critical regulatory events downstream of Notch signaling are transcriptional. Both the transcriptional downregulation of the mitotic activator *string/Cdc25*, as well as the transcriptional upregulation of *fzr*, at the mitotic/endocycle boundary are Notch dependent (Schaeffer *et al.*, 2004; Shcherbata *et al.*, 2004). Indeed, the Notch phenotype can be partially recapitulated by overexpression *string/Cdc25* in a *fzr* mutant background, supporting the model that both Fzr and Stg/Cdc25 are important mediators of the mitotic/endocycle transition in this cell type (Schaeffer *et al.*, 2004).

Having a signal from the germ line dictate the cell cycle fate of the overlying somatic cells provides an effective mechanism to coordinate the development of the two cell types that comprise the egg chamber. Yet, whether components of the Notch pathway directly influence the regulation of the cell cycle machinery in the follicle cells remains unclear. The



**Figure 2** The Notch signaling pathway controls the mitotic/endocycle switch in the follicle cells of the ovary. A late-stage egg chamber containing a mutant clone of *Su(H)* stained with DAPI (blue) to highlight nuclei, GFP (green) and  $\alpha$ -Armadillo (red). Mutant cells are marked by the absence of GFP (green) staining, while  $\alpha$ -Armadillo outlines all cells. *Su(H)* is a downstream activator of the Notch pathway, and is required cell autonomously for entry into the endocycle in follicle cells. Thus, cells in an *Su(H)* mutant clone have smaller nuclei, reflecting reduced DNA content, relative to adjacent wild-type follicle cells. Figure derived from Deng *et al.* (2001)

epistatic relationship between cell cycle regulation and differentiation is often difficult to delineate. While *Notch* mutant follicle cell clones correctly regulate some markers of differentiation, such as the Broad Complex, they incorrectly regulate others, such as *FasIII* (Deng *et al.*, 2001; Lopez-Schier and St Johnston, 2001). Thus, it is difficult to determine if Notch-dependent differentiation is required before the follicle cells are competent to enter the endocycle or if the Notch signaling pathway directly acts on the cell cycle machinery to effect the mitotic/endocycle switch. This could be accomplished, for example, by downstream components of the Notch pathway directly regulating *string/Cdc25* or *fzr* expression. What is certain is that Notch does not control the mitotic/endocycle switch in all cell types. Nurse cell endocycles are normal in *Notch* mutant clones. The precise mechanism by which Notch signaling promotes endocycles in follicle cells will be an exciting area of future research.

### Endocycle control by growth regulatory pathways

As embryogenesis and metamorphosis occur in a self-contained system (i.e. the egg and the pupae, respectively), the growth phase of *Drosophila* development occurs during the three larval stages. Egg hatching

results in a voracious first instar larvae that increases in mass 200-fold over an approximately 5-day period, culminating in pupation at the end of the third larval instar. Essentially, all dedicated larval tissues are polyploid with most of larval growth achieved via endocycles that increase cell size rather than cell number. In contrast, the imaginal discs, containing the precursor cells of adult structures such as the eyes, wings and legs, as well as the CNS grow through proliferation of diploid cells. The accumulated biomass in larval polyploid tissues is used to support the formation of adult structures during metamorphosis. Recent work on the mechanisms of growth control have shown that endocycling cells respond to the same positive and negative regulators of growth as diploid cells (Edgar, 1999; Swanhart *et al.*, 2004). Thus, the endocycle can serve as a model for how a cell integrates growth signaling with execution of the G<sub>1</sub>-S transition.

When a first instar larvae is deprived of food upon hatching, larval tissues do not initiate endocycles and remain quiescent (Britton and Edgar, 1998). When such starved larvae are provided nutrients, the endocycles resume. Thus, the endocycle responds to the larvae's environmental growth conditions. Similarly, endocycles in the polyploid nurse cells of the ovary respond to the nutritional status of the female (Drummond-Barbosa and Spradling, 2001). While this is perhaps not surprising, there are cell types, including the mushroom body neuroblasts in the larval brain, that replicate even under starvation conditions (Britton and Edgar, 1998). As described above, the E2F transcription factor and cyclin E are key regulators of endocycles. Forced expression of either *cyclin E* or *E2f1* in endocycling tissues stimulates S phase, suggesting that nutrient signaling controls the activity of these key regulators of the G<sub>1</sub>-S transition. In contrast, E2F cannot induce S-phase initiation in quiescent mitotic cells of starved larvae. Moreover, while mitotic cells continue to replicate up to 7 days after withdrawing nutrients from a well-fed larvae, endocycling cells become fully quiescent within 3 days. This indicates that the endocycle is more tightly coupled to nutritional status than the mitotic cycle. One possible explanation for this difference is that mitotically active cells in the developing CNS and imaginal discs are subjected to both nutritional and developmental cues (Caldwell and Datta, 1998; Park *et al.*, 2003), whereas endocycling cells represent differentiated cell types whose primary job is to respond to the environmental growth conditions.

Dietary amino acids provide the specific signal that controls the growth response in endocycling cells (Britton and Edgar, 1998). This response is mediated at the cellular level by the evolutionarily conserved insulin receptor (IR)/PI3K pathway (Britton *et al.*, 2002). Systemic control by the IR/PI3K pathway involves circulating insulin-like peptide hormones produced by various larval tissues, and not by direct depletion of nutrients *per se* at the cellular level (Britton and Edgar, 1998; Kawamura *et al.*, 1999; Brogiolo *et al.*, 2001). How dietary amino acids control the production of *Drosophila* insulin-like hormones, and thus the

endocycle response, is not clear. However, there is a better understanding of the cell autonomous effects of IR/PI3K signaling. In general, genetic reduction of IR/PI3K signaling inhibits the endocycle and results in small cells with reduced ploidy, whereas stimulation of PI3K signaling induces endocycle progression and results in larger than normal cells with increased ploidy (Johnston and Gallant, 2002; Hafen and Stocker, 2003). One of the key effectors of the PI3K pathway is the protein kinase TOR, which controls ribosome biogenesis and cap-dependent translation in response to amino-acid availability by phosphorylating p70S6K and 4E-BP1, respectively. Genetic manipulation of TOR or its regulators (e.g. TSC1/2 and Rheb) indicates that TOR activation stimulates growth and endocycle progression, whereas its inhibition has the opposite effect (Saucedo *et al.*, 2003; Stocker *et al.*, 2003; Zhang *et al.*, 2003). Moreover, activation of IR/PI3K signaling or TOR activity can bypass the starvation arrest and induce endo-S phase. Interestingly, this type of forced bypass results in rapid death to the animal, suggesting that the downregulation of IR/PI3K signaling and cessation of the endocycle is a necessary response to conditions of nutrient limitation (Britton *et al.*, 2002).

Other well-known regulators of proliferation, including dMyc and Cyclin D/Cdk4, affect growth in endocycling cells as well. As in mammals, *Drosophila* Cyclin D/Cdk4 can counteract the ability of pRB to inhibit E2F (Datar *et al.*, 2000; Xin *et al.*, 2002). In this way, it could play a positive role in endocycle progression. However, Cyclin D/Cdk4 is not *required* for cell cycle progression in either mitotic or endocycling cells, and its primary role in flies appears to be the regulation of growth. *Cdk4* mutant flies are viable but smaller than their wild-type counterparts, and overexpression of cyclin D/*cdk4* causes hyperplasia (accelerated cell division) in mitotically active cell populations (e.g. wing disc) and hypertrophy (increased size) in quiescent, differentiated cells (Datar *et al.*, 2000; Meyer *et al.*, 2000, 2002a). Similarly, overexpression of cyclin D/*cdk4* stimulates growth in endocycling cells resulting in an increase in final ploidy (Datar *et al.*, 2000). The mechanisms by which Cyclin D/Cdk4 controls growth are not known, but they appear not to involve the regulation of the Rb/E2F or IR/PI3K pathways. The Hph prolyl hydroxylase, which controls the response to hypoxia in mammalian cells, was recently implicated as a downstream mediator of Cyclin D/Cdk4's growth stimulating capability (Frei and Edgar, 2004). How Hph functions in this role is not known, but it may act as a metabolic sensor of O<sub>2</sub> levels necessary to support growth and endocycle progression.

*Drosophila* dMyc has similar effects on growth as Cyclin D/Cdk4. While dMyc is not absolutely required for cell proliferation, reduction of dMyc function results in poor cellular growth, while increased dMyc expression stimulates growth (Johnston *et al.*, 1999; Maines *et al.*, 2004). This effect is particularly dramatic in endocycling cell populations of both the larvae and ovary, where changes in the activity of dMyc directly result in changes of ploidy: reduced dMyc reduces

ploidy, while increased dMyc increases ploidy (Maines *et al.*, 2004; Pierce *et al.*, 2004). dMyc has many transcriptional targets that play roles in growth and cell proliferation, but precisely which targets mediate the growth response is unknown. Myc controls the expression of rRNA genes, suggesting that one way dMyc contributes to growth is via ribosome biogenesis (Grewal and Saucedo, 2004). This is reminiscent of the ability of the IR/PI3K/TOR pathway to stimulate ribosome biogenesis and translation, but dMyc apparently does not act through this pathway (Prober and Edgar, 2002). Overexpression of dMyc, or cyclin D, does not activate PI3K activity, and dMyc cannot bypass the starvation-induced growth arrest, as activity of the IR/PI3K pathway can (Britton *et al.*, 2002).

Exactly how do growth stimulating pathways impact the endocycle? Do they switch on an endocycle oscillator, and/or do they modulate the frequency of this oscillator? Overall, the data is most consistent with changes to the endocycle being secondary to changes in growth rates. That is, blocking growth will secondarily block the endocycle. Moreover, in mutants that reduce growth-like dMyc, the cyclic expression of the endocycle regulators and Dacapo remain intact (Maines *et al.*, 2004). Thus, growth rates likely modulate the rate at which the endocycle 'oscillator' runs, rather than affecting the oscillator directly. However, there is a close reciprocal relationship between cell cycle progression in the endocycle and growth, since blocking the endocycle by pRB overexpression results in reduced overall growth, perhaps because increases in ploidy ultimately are needed to support large cells (Datar *et al.*, 2000).

## Conclusions

Our view on the nature of the endocycle has evolved considerably over time. Initially, endocycling cells were considered to be cellular renegades that did not adhere to the basic principles that applied to the regulation of the mitotic cycle. However, as outlined above, recent work has shown that the endocycle provides an excellent model for examining the regulation of the G<sub>1</sub>-S program, DNA replication and the developmental regulation of the cell cycle. Indeed, the *Drosophila* endocycle is particularly well suited to address several questions fundamental to our basic understanding of the cell cycle, including the role of Cyclin E in the loading the MCM(2-7) complex onto DNA replication origins, and the regulatory mechanism underlying the oscillation of the Cyclin E protein. All available evidence indicates that the endocycle is derivative of the mitotic cycle (Edgar and Orr-Weaver, 2001). Thus, studies of this simplified cell cycle will help to delineate the core cell cycle machinery required to construct a basic cell cycle oscillator, as well as how this oscillator is regulated by both intrinsic and extrinsic inputs.

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